Some Studies in the Agglutination of Typhoid

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SOME STUDIES IN THE AGGLUTINATION OF TYPHOID.

I. Experimental Immunity and its Relation to the Agglutination Reaction.

The agglutination reaction or Widal's Test is a phenomenon well known to bacteriologists and clinicians. It is performed as follows: A "bacterial emulsion" is made usually by washing the Typhoid bacilli from the surface of a fresh culture of growing on agar medium with normal salt solution. Blood is taken from a typhoid patient or a person who has recently had typhoid. The serum is separated and made into a series of dilutions usuallyranging from a strength of one part in eight parts of normal salt solution to one part in 250 or often much higher. Equal parts of diluted serum and bacterial emulsion are then mixed together and allowed to stand for about two hours. On examining this mixture microscopically it is found that the bacilli instead of being free swimming and evenly distributed thruout the liquid, are clumped together in groups and masses, and behave exactly as if the surface of their bodies were mucilaginous. If examined with a hand lens the mixture is seen to be changed from a smooth pearl-whitish cast to a distinct flaky appearance. If given time these clumps and masses of bacilli will settle to the bottom as a whitish sediment.

As a control against possible error of observation, a sample of the bacterial emulsion unmixed with serum must alwwys be examined. It should be found to be a smooth opalescent or pearly white color with no appearance of "flakes", and should show no grouping or clumping of bacilli when examined under the microscope. Serum from a normal individual will usually cause clumping of the bacillus typhosus in dilutions of from 1:8 to 1:24. For this reason Widal's Test, in order to be diagnostic of typhoid fever, should show agglutination in a dilution of at least 1:48.

As a basis for this experiment a considerable number of students were "vaccinated" with Typhoid bacilli to give them immunity or increased resistance to the disease, after the method found so effective in the British armies in India and Africa. This was performed as follows: Bacillus typhosus was grown 24 hrs. on Agar medium at a temperature of 35°C. This growth was then washed off with normal salt solution and the bacilli killed by heating for two hours at a temperature of 65°C. The solution was then proved sterile by making both cultures from it.

The number of bacilli was estimated by the Zeiss blood-count apparatus at 1000 millions per c.c. The students were given a first dose of 1/2 c.c. or approximately 500 millions, killed bacilli as a subcutaneous injection in the abdomen. This was followed seven days later by a second injection of 1c.c. or 1000 millions of killed bacilli approximately. Only a few took a third inoculation which was given twenty one days later and consisted of approximately 2000 millions of killed bacilli.

The symptoms which were manifested after each injection were slight local redness, swelling and soreness for about 48 hours. In a few cases the lymphatics of the groin and axilla were very slightly enlarged. Almost all gave a description of headache, lassitude and general malaise beginning about four hours after the injection and lasting about twenty four hours. Some showed slight increase in pulse and temperature but all continued their school work as usual after the treatment.

On the day of the first injection a sample of blood was taken from each and the serum tested for agglutination with a live, motile, 24 hr. Agar growth of B. typhosus. Fifty three days after the first injection a second sample of blood was collected and the serum tested for agglutination as before.

The following table shows the result.

Name	Injection: 1st.	2nd	3rd.	Previous history of typhoid	Agglutinating dilution: Before:	After:
W. S.	+	+	+	10 yrs. before	1:8	1:192
M. S.	+	+	+	-- -- --	1:24	1:96
H. C.	+	+	+	-- --- --	1:4	1:96
P. C.	+	+	+	-- -- --	1:24	1:192
W. W.	+	+	+	-- -- --	1:8	1:192
E. W.	+	+	+	9 yrs before	1:12	1:256
L. F.	+	+	+	-- --- --	1:16	1:192
D. S.	+	+	--	8 yrs before	1:32	1:128
W. Q.	+	+	--	-- -- --	1:8	1:64
F. J.	+	+	--	-- -- --	1:8	1:128
E. B.	+	+	--	-- -- --	1:16	1:64
M. G.	+	+	--	-- -- --	1:12	1:64
J. L.	+	+	--	-- -- --	1:24	1:128
F. M.	+	+	--	-- -- --	1:8	1:128
H. J.	+	+	--	-- -- --	1:16	1:192
T.A.	+	+	--	-- -- --	1:16	1:96
H. O.	+	+	--	-- -- --	1:8	1:96
W. H.	+	+	--	-- -- --	1:12	1:96
M. B.	+	+	--	-- -- --	1:8	1:128
A. H.	+	+	--	-- -- --	1:16	1:96
V. M.	+	+	--	-- -- --	1:16	1:128
N. G.	+	+	--	-- -- --	1:8	1:96
N. S.	--	--	--	Control	1:16	1:16

In all cases there was a marked increase in the agglutinative power of the blood, which leads to the suggestion that in applying the Widal test for Typhoid it is well to find out if the patient has ever been inoculated against Typhoid. Otherwise a positive Widal test might lead to an error of diagnosis.

It is known that the agglutinative power of the blood does not run exactly parallel with the individual's resistance to Typhoid, However it may be taken as a rough index to the patient's immunity. This being the case, all those treated received a considerable increase of resistance to Typhoid as indicated by the above test. How effective

this may prove, and for what time it may persist will be interesting to follow. It is of interest to note that the three students who had a previous history of Typhoid some years before, had no higher agglutinative power than the others, which would indicate that the immunity acquired by having the disease is not lasting.

II. An Attempt to Modify the Agglutinability of Bacillus Typhosus by Selective Isolation of Individual Bacilli.

All observers have noted in performing agglutination experiments, even with highly agglutinative serum in low dilutions, that some individual bacilli resist agglutination and remain floating free in the serum. The object of this series of experiments was to determine whether this failure to clump is due to some peculiar property which can be transmitted to subsequent cultures.

Method. To isolate individual bacilli selectively seemed necessary as a means for this investigation. The most precise method for accomplishing this selective isolation is the technique designed and developed by Dr. M.A.Barber of the University of Kansas.
(See Journal of Infectious Diseases. Vol.V p. 380, Vol. VIII. p.348.
Kansas University Science Bulletin 1907 p. 8.).
By using a microscopic capillary pipette supported in a mechanical holder provided with motion in three directions, one is able after considerable practice to isolate individual micro-organisms from a hanging drop. This being supported by a cover glass, OVER A MOIST CHAMBER which is held and moved about under the microscope by the ordinary mechanical stage. The details of the method are fully described in the articles above referred to.

The medium used was meat broth carefully made according to the American Public Health Association standard, reaction 0.7 acid to phenolphthalein. To begin with, an absolutely pure strain of B. typhosus was obtained by isolating an individual bacillus from a culture obtained ~~by~~ from the laboratories of Johns Hopkins University. The resulting colony was transferred to broth and several Agar slants were inoculated from this and labeled "Stock". A drop of the broth culture was placed on the sterile cover glass, and agglutin-

ation performed by adding an equal part of serum diluted 1:16. The serum used thruout this experiment was human serum from the same individual OBTAINED under aseptic conditions and proved sterile by broth culture. It agglutinated Typhoid bacilli at 1:96 dilution. As soon as clumping began a single definite clump was selected and isolated by means of the capillary pipette. Then several individual bacilli which had resisted clumping were isolated and planted separately in small hanging drops. Chemically clean glass tubing was used for making all pipettes. For making each transfer of cultures, broth, or serum, and for each operation of isolation a new freshly drawn pipette was used. The cover glass was then transferred to a vaseline sealed hollow-ground slide of large size and incubated twenty four hours at 35°C. From this time on a double series of isolations and cultures was made. In the one series the first clumps to form were isolated, and in the other the individuals which resisted agglutination were selected and allowed to form colonies. For convenience we will refer to these as +S and -S respectively, the +S referring to that series selected on the basis of ready clumping and the -S to the series developed by selecting the resistant individuals. The number following the S indicates the number of selections made in that series.

After making five selections and cultures of the +S and four of the -S a preliminary comparative agglutination test was made with results as shown in the following table.

Dilution:	1:16	1:24	1:24	1:32	1:48	1:96	Control
+S5	+	+	+	+	+	?	--
--S4	+	+	+	+	+	--	--

In making this test twenty four hour cultures were used and the dilutions were examined microscopically. The writer in this case knew the identity of the cultures but in all subsequent tests the cultures were marked in cipher by a second person and their identity revealed only after all observations had been made and recorded. One element of the personal equation was thus eliminated. The following table shows the result of a test in which the original stock was compared with +S9 and --S8.

Dilutions:	1:32	1:48	1:64	1:96	1:128	1:192	1:250	Control
+S9	+.	+.	+.	+.	+.	+	--	--
--S8	+.	+.	?	--	--	--	--	--
Stock	+.	+.	+.	+.	--	--	--	--

These results were verified by a second observer before the identity of the cultures was revealed. The test shows a marked increase in the agglutinability of the +. series and a corresponding decrease in the agglutinability of the negative series.

Following this test the routine of performing agglutinations and selections was continued until 24 selections in the +S and 13 in the --S had been made. The average time of making the +. selections was twenty minutes, while the -- series were exposed to the action of the serum for two hours before the selections were made. Frequent transfers were made to clean, freshly sterilized cover glasses.

The following test was made with 18 hour Agar cultures using rabbit serum which agglutinated at about 1:600 In this test the macroscopic method was used and the observations were made after three hours at room temperature, 26°C.

Dilut.	1:64	1:96	1:128	1:192	1:256	1:384	1:512	1:768	Control
+S24	++.	++.	++.	++	++.	++.	++.	++.	--
--S13	++.	++.	++.	++.	++.	+.	+	--	--
Stock	++.	++.	++.	++.	++.	++.	+	--	--

++. means agglutination visible to the naked eye.
+. " " " with hand lens.

Following this test the cultures were kept at room temperature and transferred to fresh media three times in eighteen days. Then a final test was made macroscopically, using both rabbit serum and the same human serum which was used in developing the two series. The cultures were marked in cipher as before.

Rabbit Serum.

Dilut.	1:250	1:384	1:500	1:768	1:1000	1:1500	1:2000	Control
+S24	++.	++.	++.	++.	++.	+.	+.	--
--S13	++.	++.	++.	++.	++.	+.	+.	--
Stock	++.	++.	++.	++.	++.	+.	--	--

Human Serum.

Dilutions:	1:16	1:24	1:32	1:48	1:64	1:96	1:128	Control
+S24	++.	++.	++.	++.	+.	+.	--	--
--S13	++.	++.	++.	++.	++.	++.	+.	--
Stock	++.	++.	++.	+.	+.	+.	0.	--

Conclusions: The first two tests showed quite decided differences in the agglutinability of the two series. However in the final tests no such difference was evident. In the one the --S13 agglutinated more readily than the +S24, and in the other test the --S13 agglutinated more readily than the stock. So we must conclude that, whatever differences in agglutinability were developed, they failed to become permanent or even to persist thru the number of generations which would take place in Agar media in eighteen days at 26°C.